Desmuslin gene knockdown causes altered expression of phenotype markers and differentiation of saphenous vein smooth muscle cells

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Objective: **Phenotypic alterations of vascular smooth muscle cells (VSMCs) appear critical to the development of primary varicose veins. Previous study indicated desmuslin, an intermediate filament protein, was differentially expressed in smooth muscle cells (SMCs) isolated from varicose veins; thus, it was naturally hypothesized that altered desmuslin expression might in turn affect the functioning of VSMCs, leading to the phenotypic alterations and varicose vein development.**

Methods: **In this study, expression of desmuslin in normal human saphenous vein SMCs was knocked down using small interfering RNA (siRNA), and control cells were treated with a scrambled siRNA sequence. The levels of several phenotypic markers including smooth muscle (SM) α-actin and smooth muscle myosin heavy chain (SM-MHC) were assessed. Collagen formation, matrix metalloproteinase expression (MMP-2), and cytoskeletal and morphological changes were also examined.**

Results: **SMCs treated with desmuslin siRNA exhibited significantly increased levels of collagen synthesis and MMP-2 expression and decreased expression levels of SM α-actin, SM-MHC, and smoothelin and exhibited disassembly of actin stress fibers when compared with the control cells. Changes in cell morphology and actin fiber networks in VSMCs treated with desmuslin siRNA were consistent with a lower degree of differentiation.**

Conclusions: **These results indicated desmuslin expression is required for the maintenance of VSMC phenotype. Decreased desmuslin expression may affect differentiation of VSMCs and ultimately contribute to the development of varicose veins. (J Vasc Surg 2010;52:684-90.)**

Clinical Relevance: **Primary varicose veins are a frequent and refractory disease of the peripheral veins. Improved understanding of the cellular and molecular mechanisms involved may allow the identification of additional targets for pharmacologic intervention.**

The development of primary varicose veins is the most common disease of peripheral veins and affects 10% to 40% of individuals between the ages of 30 and 70 years.[1] The mechanisms underlying the pathogenesis of this condition remain unclear.

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Supported by the National Natural Science Foundation of China (Grant No. 30471685), the Natural Science Foundation of Guangdong Province (Grant No. 071173650), and the First Affiliated Hospital of Sun Yat-sen University and Academy of Life Science (Grant No. 162006).

Competition of interest: none.

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The editors and reviewers of this article have no relevant financial relationships to disclose per the JVS policy that requires reviewers to decline review of any manuscript for which they may have a competition of interest.

0741-5214/$36.00

doi:10.1016/j.jvs.2010.03.069

The principal function of mature vascular smooth muscle cells (VSMCs) is contraction. These cells have a high cytoplasmic volume fraction of myofilaments,[2,3] a low rate of proliferation,[4] and synthesize only small amounts of matrix proteins.[5] VSMCs are not terminally differentiated cell, and have been shown to undergo phenotypic switching in association with various abnormal physiological conditions. More importantly, the differentiation of VSMCs has been implicated in various vascular pathologies, including atherosclerosis,[6,7] intimal hyperplasia,[8,9] and varicose vein formation.[10-14]

Several studies have described phenotypic alterations of VSMCs in varicose veins.[10-14] Specifically, it has been reported that VSMCs isolated from varicose veins exhibit phenotypic transformation from a contractile to a synthetic state.[10-14] Electron microscopic analysis of these cells further revealed that they were poorly differentiated, with an increased presence of secretory cytoplasmic organelles (suggesting unusual synthetic and secretory roles for these VSMCs) and a reduction in filament bundles (implying decreased contractile capability).[10]

It is well established that there is a genetic component to the formation of varicose veins.[15-17] Indeed, differentially expressed genes in VSMCs isolated from varicose veins have been identified.[18-21] These genes consist of extracellular matrix (ECM) molecules and cytoskeleton proteins.

Previous studies have shown that matrix metalloproteinase-2 (MMP-2) plays a pivotal role in regulating and rearranging the ECM components of the venous wall.[22] Moreover, findings from recent studies raise the interesting possibility that increased MMP-2–induced venous relaxation can lead to progressive venous dilatation, chronic venous insufficiency, and varicose vein formation.[23,24]

Intermediate filament (IF) protein, in combination with microtubules and microfilaments, constitutes the cytoskeleton of SMCs. IF proteins consist of a family of proteins that share common structural and sequence features. The IF network has previously been regarded as a fixed cytostructure that solely provides mechanical integrity to the cell.[25] However, it is now known that not only do IFs form coordinated interactions with other cytoskeletal elements and organelles, but they also interact with noncytoskeletal proteins (eg, chaperones, enzymes, receptors, etc.) forming networks that can affect diverse cellular functions.[26] Furthermore, recent studies demonstrate that the IF cytoskeleton is a dynamic signaling platform involved in cell growth, differentiation, and other fundamental biological behaviors.[27,28]

In a previous study, we found that desmuslin, a type VI IF protein[29] and a component of the extensive cytoskeletal network, was significantly downregulated in SMCs isolated from varicose veins.[18] This led us to hypothesize that altered desmuslin expression may deleteriously affect the functioning of venous wall SMCs, leading to phenotypic alterations and contribute to the development of varicose veins. To begin to investigate this possibility, we isolated SMCs from normal human saphenous veins and examined the expression levels of a number of VSMC phenotype markers (including smooth muscle [SM] α-actin, SM myosin heavy chain [SM-MHC], and smoothelin) after desmuslin gene knockdown using small interfering RNA (siRNA).[30,31] Collagen formation, MMP-2 expression, and morphological changes were also assessed.

MATERIAL AND METHODS

VSMCs. Smooth muscle cells were isolated from human saphenous veins. Twelve veins were obtained from patients undergoing coronary bypass surgery. The patients were eight men and five women with a mean age of 58.7 ± 1.2 years (range, 52-68 years). Preoperative evaluation of these patients revealed an absence of varicose abnormalities.

All patients provided informed consent prior to the procedure and the study was approved by the ethics committee of the First Affiliated Hospital of Sun Yat-sen University. The investigation conformed with the principles outlined in the Declaration of Helsinki.

Table 1. Oligo siRNA sequences used for desmuslin gene knock-down

Oligo 1: sense 5′	3′GCACACGGUCAAAUGAGAGTT
anti-sense 5′	3′CUCUCAUUUGACCGUGUGCTT
Oligo 2: sense 5′	3′GCCUUACCAUGCAUUUCCGTT
anti-sense 5′	3′CGGAAAUGCAUGGUAAGGCTG
Scramble: sense 5′	3′GUGAGACCGUAAACGCAATT
anti-sense 5′	3′UUGCGUUUACGGUCUCACTT

Cell culture. Explants from the medial layer of saphenous veins were prepared as previously described.[12] To avoid contaminating the preparation with fibroblasts, we carefully removed the adventitia and then scraped the luminal surface to remove the endothelial cells. Furthermore, after cutting the vessel into 2- × 2- mm explants, we placed the explants with the luminal side down in a Petri dish. Cells were grown in 25-mL flasks in Dulbecco's modified Eagle medium (Hyclone; Thermo Scientific, Waltham, Mass) supplemented with 10% fetal bovine serum (Hyclone), 2 mmol/l L-glutamine, 10^5 U/L penicillin, 100 μg/mL streptomycin, and 100 μg/mL nonessential amino acids. Incubation conditions were: 37°C, 95% O^2, and 5% CO_2. The culture medium was changed every 2 days. Cell growth began within 7 to 14 days, with confluence being attained after 4 weeks. After attaining confluence, cells were trypsinized and seeded at densities of 10,000 cells/cm^2 (first passage) in uncoated culture flasks. Further subculturing was performed. Only cells from passages 0 to 2 were used for experimentation. Before each experiment, the SMCs were confirmed by immunofluorescence using a monoclonal antibody against human α-actin (clone ASM-1; Chemicon International Inc, Billerica, Mass).

SMCs from each donor were separately cultured and studied as outlined in the following sentence. Desmuslin knockdown studies were performed on cells obtained from every patient, while phenotype marker assessment and MMP-2 expression was assessed in cells obtained from five patients, collagen synthesis in cells obtained from four patients, and morphology in cells obtained from three patients.

Transfection of VSMCs with short interfering RNA. Two 21-nucleotide siRNA sequences targeting human desmuslin at exons 4 and 1 were synthesized (Ambion Inc, Austin, Tex) on the basis of mRNA sequences NM-015286 and NM-145728. A scrambled siRNA sequence with the same nucleotide composition as desmuslin siRNA, but lacking significant sequence homology to the human genome, served as an experimental control. The oligo siRNA sequences can be seen in the Table. VSMCs were seeded into 6- or 24-well plates and, upon attainment of 80% to 90% confluence, were transfected with siRNA using Lipofectamine 2000 (Invitrogen, Carlsbad, Calif) according to the manufacturer's procedure. The subsequent transfection efficiency was estimated by Western blotting.

Western blotting. Protein extracts were obtained by disrupting the transfected cells in lysis buffer. Supernatants containing cytoplasmic proteins were collected and

the protein concentrations determined using the bicinchoninic acid method (Protein Assay Kit; Keygen Biotec, Nanjing, China). For each sample, 50 μg of total protein was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis on a 6% to 12% polyacrylamide gel. Separated proteins were electrophoretically transferred onto polyvinylidene fluoride membranes for immunodetection. After blocking with 5% non-fat milk in 0.02 M Tris and 0.05% Tween 20, membranes were incubated with the following primary antibodies diluted in blocking buffer: anti-α-SM-actin (clone ASM-1), anti-myosin smooth muscle heavy chain, anti-smoothelin (Chemicon International Inc.), anti-MMP-2, and anti-desmuslin (Santa Cruz Biotechnology, Inc). Thereafter, samples were incubated with horseradish peroxidase-conjugated secondary antibodies. Detected proteins were visualized by enhanced chemiluminescence system (Amersham, Otelfingen, Switzerland). Band densities were quantified and normalized with reference to α-tubulin (Sigma-Aldrich, St. Louis, Mo).

Collagen synthesis assay. Culture media containing 50 μGi/ml L-[3,4-^{3}H] proline (China Institute of Atomic Energy, Beijing, China) was added to each well containing transfected cells. After 48 hours of incubation, media were removed from the cells. Trichloroacetic acid (TCA) was then added to the cells at a concentration of 10% and the cells left on ice for 1 hour. Following centrifugation at 3000 × *g* for 30 minutes, the precipitated protein was washed with 4 mL of ice-cold 10% TCA to remove any unincorporated labeled proline and then re-centrifuged. The supernatant was carefully removed and the pellet resuspended in 0.3 mL of 0.3 M NaOH/0.3% sodium dodecyl sulfate. Preparations were then warmed to 37°C until solubilized, and 4 mL of liquid scintillant was added. Radioactivity was counted in both the media and cell layer samples using a liquid scintillation analyzer. The amounts of radioactivity incorporated into soluble and insoluble extracellular protein fractions provide an assessment of collagen released into the media (soluble protein) relative to that released and then incorporated in and around the cell layer (insoluble protein).[32]

Assessment of VSMC by Immunofluorescence staining. Immunofluorescence staining was performed to detect cytoskeleton changes in transfected VSMCs. Cells (2×10^3) on coverslips were washed in phosphate-buffered saline (PBS) and fixed in methanol at −20°C for 10 minutes. The cells were then preincubated in 10% bovine serum albumin for 10 minutes at room temperature to block nonspecific binding. Following this, the cells were incubated with a monoclonal antibody against human α-actin (clone ASM-1; Chemicon International Inc) diluted in a solution containing 1 × PBS and 0.1% Triton X-100 for one hour at room temperature with shaking. After washing with PBS three times (10 minutes each wash), cells were incubated with the appropriate combination of secondary antibodies (FITC-conjugated anti-goat IgG [H+L] or rhodamine-conjugated anti-mouse IgG; Jackson Immunoresearch, Baltimore, Md) with gentle shaking for 30 minutes. Following three washes with PBS, cells were treated with DAPI to facilitate detection of nuclei. The cells were mounted in fluorescent mounting medium (Invitrogen) and then examined by immunofluorescence microscopy.

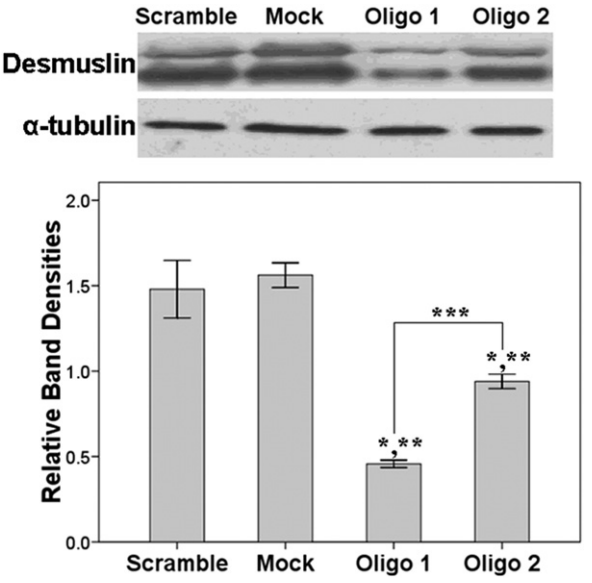

Fig 1. Desmuslin downregulation by short interfering RNA (siRNA) in vascular smooth muscle cells (VSMCs). VSMCs were transfected with desmuslin siRNA oligos 1 or 2 or siRNA scramble. A subset of cells was also mock transfected. Desmuslin protein levels were assessed by Western blot and normalized with reference to α-tubulin levels. Representative blots are shown. The results are presented as mean ± standard deviation as determined from three independent experiments (ie, cells were obtained from three different patients). *$P < .05$ vs scramble; **$P < .05$ vs mock; ***$P < .05$ vs oligo 1.

The assembly of the actin cytoskeleton into parallel and elongated stress fibers is a hallmark of differentiated VSMCs.[33] Hence, immunofluorescence-labeled transfected cells were examined under a Zeiss confocal microscope, and images were captured using Zeiss lasersharp software. All experiments thus described were performed at least in triplicate.

Statistics. Data are presented as mean ± standard deviation or median (interquartile range). Betweengroup comparisons were made by one-way analysis of variance. Post-hoc assessments were made by Bonferroni approach or Wilcoxon-rank sum test depending on the distribution. Analyses were performed using SAS 9.0 statistical software (SAS Institute Inc, Cary, NC). A P value of $<.05$ was considered statistically significant.

RESULTS

Expression of phenotype markers in siRNA-treated VSMCs. Two siRNA oligos were tested for knock-down of desmuslin expression in VSMCs. The two bands (210 KDa and 180 KDa) are the two desmuslin isoforms (Fig 1). Of these siRNA oligos, transfection with oligo 1 resulted in

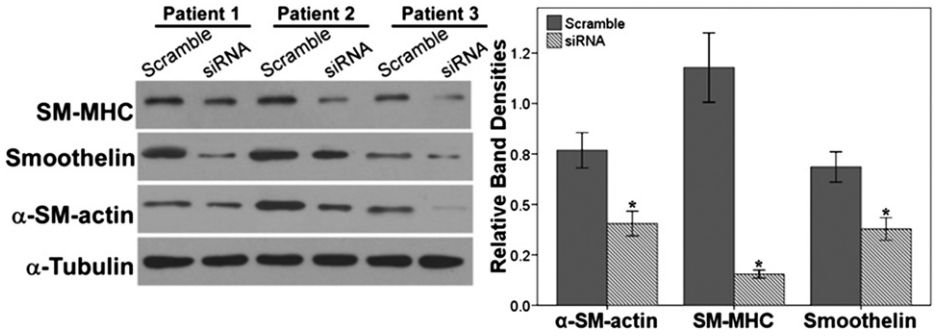

Fig 2. Expression of vascular smooth muscle cell phenotype-dependent markers (smooth muscle [*SM*] α-actin, smooth muscle myosin heavy chain [*SM-MHC*], and smoothelin) following desmuslin gene knockdown by short interfering RNA (*siRNA*). Control cells were transfected with siRNA scramble. Protein levels were assessed by Western blot and normalized with reference to α-tubulin levels. Representative blots are shown. The results are presented as mean ± standard deviation as determined from five independent experiments (ie, cells were obtained from five different patients). **P* < .05 vs scramble.

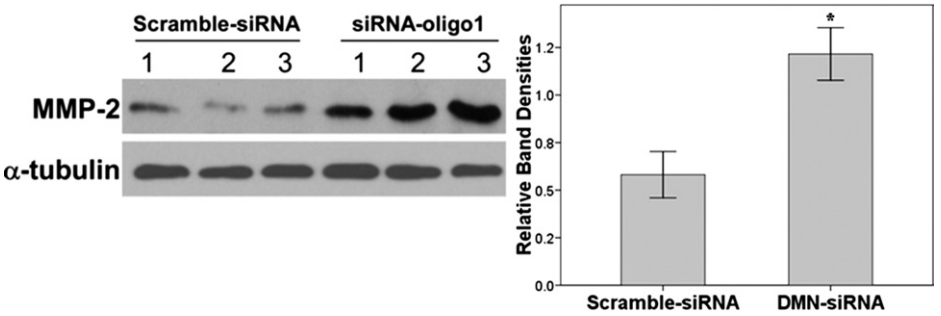

Fig 3. Expression of matrix metalloproteinase-2 (*MMP-2*) in vascular smooth muscle cells following desmuslin gene knockdown by short interfering RNA (*siRNA*). Control cells were transfected with siRNA scramble. Protein levels were assessed by Western blot and normalized with reference to α-tubulin levels. Representative blots are shown. The results are presented as mean ± standard deviation from five independent experiments (ie, cells were obtained from five different patients). **P* < .05 vs scramble.

greater inhibition than oligo 2; hence, oligo 1 was used in all further experimentation.

The protein expression levels of α-SM-actin, SM-MHC, and smoothelin in VSMCs transfected with desmuslin siRNA were assessed by Western blot. Significantly lower protein expression levels of each of these markers were apparent in siRNA-treated cells compared with control (siRNA scramble-treated) cells (all $P < .05$; Fig 2).

Protein expression levels of MMP-2 were significantly increased in the desmuslin-siRNA-transfected cells compared with control-siRNA-transfected cells ($P < .05$; Fig 3).

Collagen synthesis in siRNA-treated VSMCs. Incorporation of L-[3,4-^{3}H] proline into the cell layer (insoluble fraction) and in the medium (soluble fraction) of VSMCs was significantly increased (three- and four-fold) following inhibition of the desmuslin gene (both $P < .05$; Fig 4).

Morphology of siRNA-treated VSMCs. Confocal microscopic examination of desmuslin siRNA-treated VSMCs stained for actin revealed disassembly of actin stress fibers (Fig 5). Cells transfected with control siRNA had parallel and dense actin filaments characteristic of differentiated VSMCs, while cells treated with desmuslin siRNA had dispersed and thinner filaments. Cells transfected with control siRNA retained a normal, spindle-like morphology, while cells transfected with desmuslin siRNA were polygonal in shape. Desmuslin-knockdown cells were also observed to be much larger than control cells.

DISCUSSION

In the present study, we tested the hypothesis that desmuslin expression may affect the functioning of VSMCs, causing alterations in phenotype. Using siRNA to knockdown the expression of desmuslin, we found that expression levels of smooth muscle-specific structural proteins including SM α-actin, SM-MHC, and smoothelin decreased significantly, while the synthesis of total collagen increased remarkably. These previously unreported findings suggest that desmuslin may play a role in controlling the differentiation state of VSMCs.

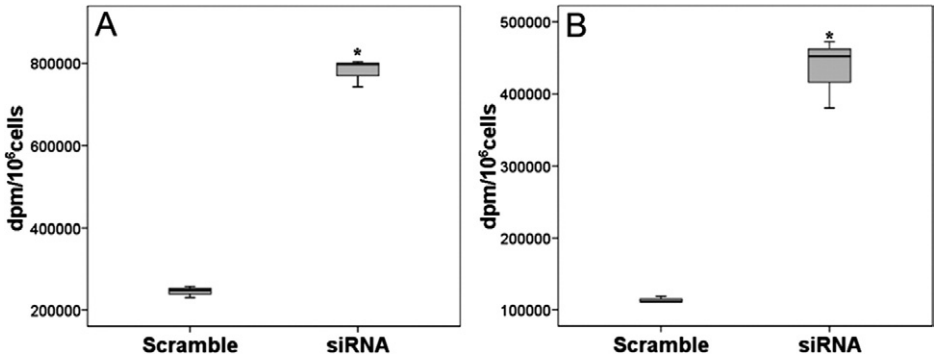

Fig 4. Collagen synthesis in vascular smooth muscle cells after desmuslin gene knockdown by short interfering RNA (*siRNA*) as indicated by incorporation of L-[3,4-^{3}H] proline into the cell layer (**A**) and media (**B**). Control cells were transfected with siRNA scramble. The results are presented as the median and interquartile range as determined from four independent experiments (ie, cells were obtained from four different patients). $*P < .05$ vs scramble.

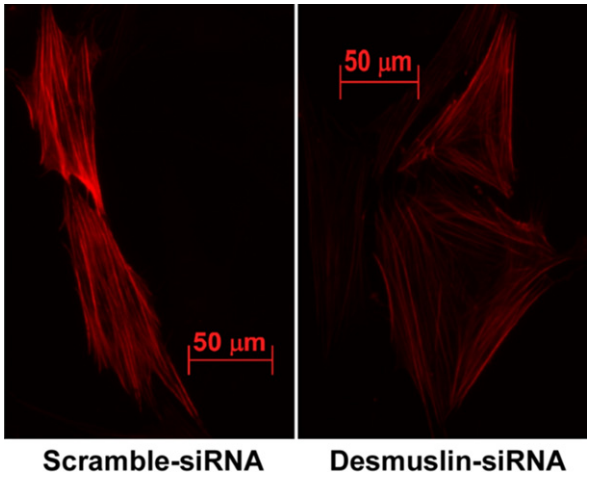

Fig 5. Representative immunofluorescence micrographs of vascular smooth muscle cells transfected with scramble small inhibitory RNA (*siRNA*) and desmuslin siRNA. Cells transfected with siRNA scramble exhibited parallel and dense actin filaments, while cells treated with the desmuslin siRNA had dispersed and thinner filaments. Cells transfected with siRNA scramble retained a normal spindle-like morphology, while cells transfected with desmuslin siRNA were more polygonal in shape. Desmuslin-knockdown cells were larger than control cells.

It has been demonstrated that the expression of contractile proteins (including SM-MHC) is necessary for optimal force development/contraction in SMCs,[34,35] and that increased expression of SM-MHC correlates with a greater Vmax of contractile shortening in these cells.[36] Furthermore, decreased expression of SM α-actin has been shown to correlate with decreased contractility.[37] The findings from these studies indicate that proper contraction of SMCs requires expression of the full complement of contractile proteins, and that alterations in expression contribute significantly to changes in the contractile properties of these cells.

Contractility is essential for the maintenance of vessel wall tone in human saphenous vein smooth muscle cells.[38,39] Indeed, impaired contractility of VSMCs has been previously shown to be associated with the development of varicose veins.[10] In the present study, we found that the expression levels of contractile proteins (including SM-MHC) were significantly decreased in VSMCs transfected with desmuslin siRNA. Although we did not make any assessments of contractility, these observations suggest that altered desmuslin expression may result in decreased contractility of VSMCs. This altered contractility may, in turn, contribute to the pathogenesis of varicose veins.

We observed that treatment of VSMCs with desmuslin siRNA resulted in dramatic cell morphological changes. Specifically, cell shape changed from spindle-like to polygonal, and reorganization of the actin cytoskeleton, an important mediator of cell contractility, was apparent. This finding is consistent with that from a report indicating that IFs play a central role in maintaining cytoarchitecture.[27] Indeed, VSMCs treated with desmuslin siRNA in the present study exhibited an unstructured network of actin filaments, with the filaments being dispersed and thinner compared with those in control cells. Coincidentally, we also found that expression of MMP-2 was significantly increased in VSMCs transfected with desmuslin siRNA. As previously noted, MMP-2 plays an important role in regulating and rearranging the ECM components of the venous wall.[22] Hence, increased MMP-2 expression in the present study may have contributed to the observed alterations in morphology. Such changes would presumably impact cell contractility. Further studies are required to assess this possibility.

Despite compelling evidence showed that phenotypic modulation of the SMCs plays a key role in varicose vein development and that desmuslin gene silencing evokes de-differentiated phenotypic switching, relatively little is known about how this process is regulated in vivo. One way to investigate the mechanisms through which inhibition of desmuslin causes SMCs phenotype is to determine whether

desmuslin interacts with and affects signaling pathways previously shown to regulate SMCs differentiation. For instance, it is well documented that RhoA activity is crucial for regulating SMCs differentiation and the assembly of actin stress fibers and F-actin.[40] Furthermore, evidence is now emerging that desmuslin influences some cell responses by modulating the dynamics of actin.[41] Thus, further investigation regarding the physical and functional interaction between desmuslin and RhoA is warranted to determine how desmuslin regulates the differentiation of SMCs.

To our knowledge, little is known about how desmuslin maintains the structural integrity of SMCs. A previous study has shown, however, that desmuslin helps maintain the integrity of skeletal myofbers by affecting expression of alpha-dystrobrevin, desmin, and other structural proteins.[42] Desmuslin may act in a similar manner in SMCs.

This study has a number of limitations that warrant mention. As already noted, we did not assess contractility of the transfected VSMCs. Given the changes reported herein, we would presume that consequential changes in contractility would be apparent. Further studies are warranted to confirm this. Further studies are also needed to elucidate the precise molecular mechanisms through which desmuslin influences expression of the various factors examined in the present study. Such characterization is yet to be reported. Animal studies (including those involving knockout mice) should also be performed to confirm the findings in an in vivo setting. We did not make any between-gender comparisons in this study. As varicose vein development is more common in women, it would be interesting to compare VSMC desmuslin expression between men and women. To our knowledge, such a comparison has not been made to date. A final limitation is the fact that saphenous veins were obtained from patients older than most patients who develop varicose veins. Desmuslin expression should be assessed in VSMCs obtained from younger patients to determine whether there are any age-related changes.

In summary, this is the first detailed in vitro study to demonstrate that phenotypical changes can be induced in VSMCs by altering expression of desmuslin. Our findings suggest that desmuslin expression is essential for the maintenance of the differentiation state of VSMCs. Low or insufficient expression of desmuslin may affect the differentiation of VSMCs leading to decreased contractility, potentially contributing to the development of varicose veins.

AUTHOR CONTRIBUTIONS

Conception and design: YX, ZH, HY, SW
Analysis and interpretation: YX, ZH
Data collection: YX, ZH, HY, HZ
Writing the article: YX, ZH
Critical revision of the article: YX, ZH, SW
Final approval of the article: YX, ZH, HY, HZ, SW
Statistical analysis: YX, ZH
Obtained funding: HY, SW
Overall responsibility: SW
YX and ZH contributed equally to this work.

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Submitted Feb 2, 2010; accepted Mar 28, 2010.